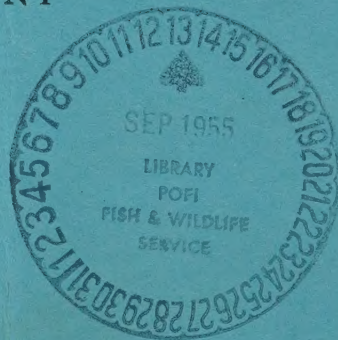




SCOTTISH HOME DEPARTMENT

Marine Research
1955 No. 3



Herring Rearing—I

The Storage of Herring Gametes

By J. H. S. BLAXTER, B.A.

EDINBURGH: HER MAJESTY'S STATIONERY OFFICE
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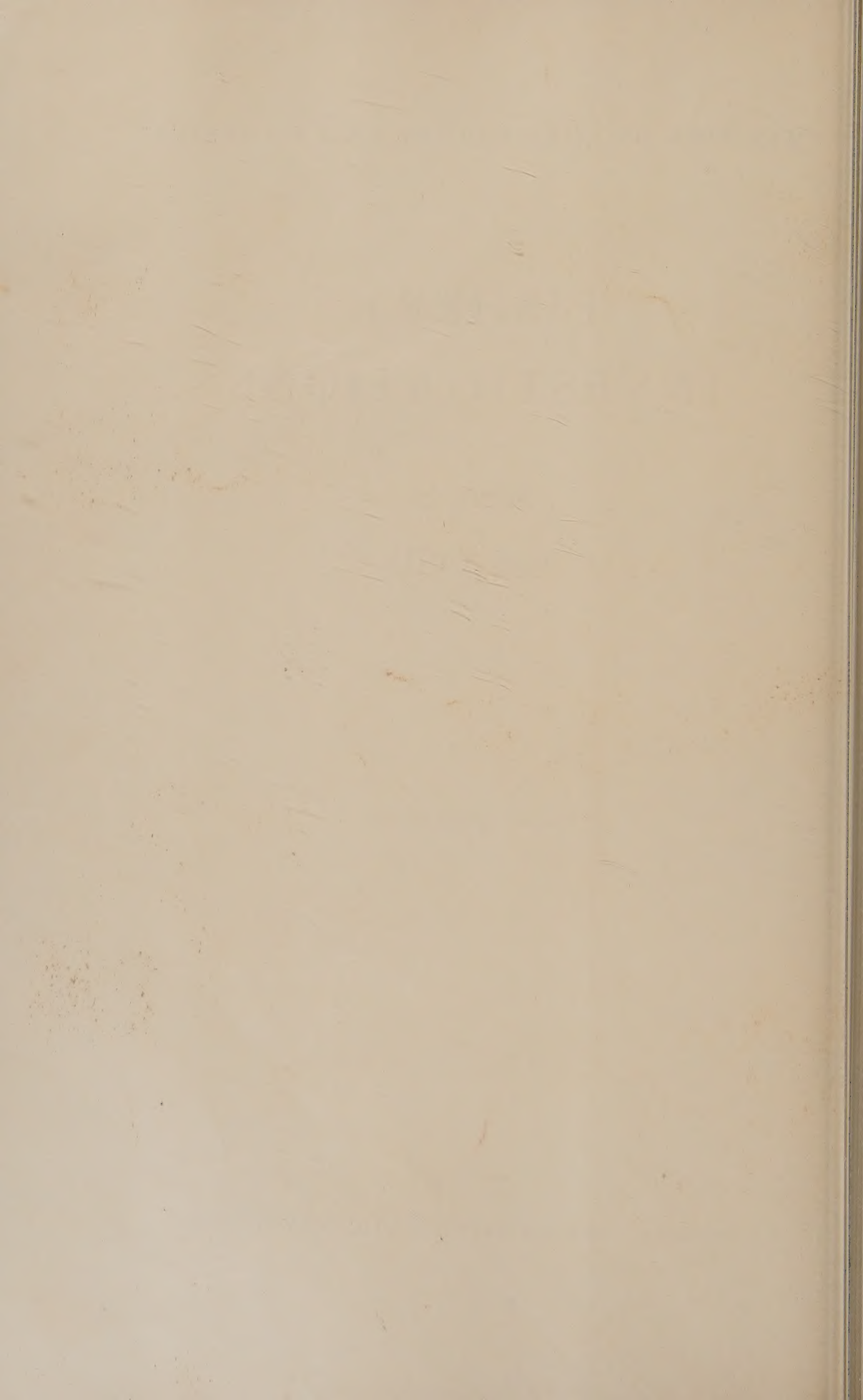
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The Storage of Herring Gametes

INTRODUCTION

In recent work on the 'racial' problem in the Atlantic herring (*Clupea harengus*, L.) it was found desirable to store herring gametes for both long and short periods of time.

A short term storage method was required which would permit catching and killing ripe herring at sea and storing the gametes for subsequent fertilisation and hatching in the laboratory under controlled conditions. This would overcome the difficulty of transporting ripe herring alive in tanks or setting up suitable apparatus under difficult conditions at sea.

Long term storage was required for crossing the two main groups of herring, one of which spawns in spring and the other in the autumn.

SHORT TERM STORAGE

PREVIOUS WORK

Fish gametes do not live for long in the untreated dead parent's body. Rutter (1902) in the Quinnet salmon (*Oncorhynchus tshawytscha*) and Nakano and Nozawa (1925) in the salmonid (*Oncorhynchus masou*) found that both eggs and spermatozoa remained fertile up to five hours after death, while Stroganov (1938) reported that the spermatozoa of the Volga herring (*Caspiolosa volgensis*) were infertile after one hour. Brook (1885) concluded that the spermatozoa of the herring (*Clupea harengus*) lived for only three hours after the male's death but that the eggs lived for 48 hours.

When semen is diluted a phase of activity is usually quickly superseded by exhaustion. This widespread 'Dilution Effect' has been reported for various freshwater fish such as the wall-eyed pike (Reighard, 1893), salmonids (Huxley, 1930; Ellis and Jones, 1939) and the Volga herring (Stroganov, 1938).

For effective storage the gametes must be removed from the adult and stored without dilution or, if diluted, the diluent must contain some factor which prevents this rapid exhaustion. In fish, gamete storage has nearly always been carried out by the "dry" (undiluted) method and has had a practical application in fish culture work. Both eggs and spermatozoa have been stored. The word "spermatozoa" is used here rather than "semen" or "milt" as the gametes have usually been obtained by dissection.

Vrassi (quoted by Soudakevicz, 1874) was probably the first worker to report on the storage of fish spermatozoa. He found that the milt of the perch (*Acerina vulgaris*) was partially fertile after four days. Atkins (1874) showed that the eggs of the Atlantic salmon (*Salmo salar*) kept in contact with air, were fertile after four days. Nakano and Nozawa (1925) and Kawajiri (1927) stored the eggs and spermatozoa of the salmonid (*Oncorhynchus masou*) at different temperatures and found lower ones more effective. Butcher (1944) stored the spermatozoa of trout under liquid paraffin at 2.2-3.3° C and found them fertile after five days and Barrett (1951), working on the Chum salmon (*Oncorhynchus keta*) found that the eggs could be stored dry for 108 hours with less than 20% loss in fertility and the spermatozoa for 36 hours with an average loss of fertility of less than 10%.

The gametes of herring have not been studied to any extent. Brook (*loc. cit.*) has studied the development of *Clupea harengus*. Williamson (1938) and Yanagimachi and Kanoh (1953) describe the manner of fertilisation in *Clupea harengus* and the Pacific herring (*Clupea pallasii*) respectively. Yanagimachi (1953) also tested the fertility of herring gametes when stored in different concentrations of Ringer solution and sea water. He found that fertility was maintained to a small extent for 12 hours in sea water at 0° C-10° C. In M/8 and M/4.5 Ringer or in 50% and 25% sea water, fertility was retained for 24 hours or longer.

Storage of semen in diluents has been carried out principally in mammals as part of research into methods of artificial insemination. The first diluents used consisted of blood serum, sugars, gelatin, buffers, etc. (see Mann, 1954) but a considerable advance was made when Phillips and Lardy (1940) used egg yolk combined with a buffer.

METHODS

The present work on the herring has included storage dry and in diluents. It was carried out as a part of a programme of herring rearing under controlled conditions.

In view of previous work it was decided to try four different methods of storage: (1) dry; (2) in water; (3) in buffered sea water, and (4) in buffered sea water and egg yolk.

The citrate and phosphate buffers used in artificial insemination did not appear to be very satisfactory as the pH values required lie between 6.5 and 7.0. It seemed more logical to use a pH value for herring gametes somewhere near that of sea water (pH 8.0). Phosphate and citrate buffers are near the extreme of their range at pH 8.0 and their buffering capacity is therefore reduced. In view of this a borate and a phosphate buffer were used.

All buffers were made up in diluted sea water (two parts sea water : one part distilled water). M/20 borate buffer was made up by adding 50 cc. M/5 H_3BO_3 to 4 cc. M/5 NaOH and then adding diluted sea water to make the volume up to 200 cc. A M/15 phosphate buffer was made by adding 95 cc. M/15 $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ to 5 cc. M/15 KH_2PO_4 (see Clark, 1928). These solutions were kept in stock and when required sea water was added to them to make the buffers about M/100.

The buffered egg yolk diluent was made when required by adding egg yolk to M/100 buffered sea water in the ratio 1 : 5.

Throughout the experiment precautions were taken to keep the gametes sterile. The sea water was filtered through a Doulton filter candle and boiled. All apparatus was autoclaved at 15 lbs. sq. in. for at least 20 minutes before use and later dissecting instruments were sterilised in an oven.

The gametes were stored in $\frac{1}{4}$ lb. honey jars with screw caps and rubber washers or in plastic top tubes. Some of these receptacles were kept in broken ice and wood shavings in a vacuum flask, which gave a storage temperature of about 4°C , and others in an open container which provided a storage temperature of about 7°C .

The herring used in the experiments were caught by gill nets on the spawning grounds in the Firth of Clyde (spring) and off the east coast of Scotland (autumn).

Ripe fish were recognised by their 'running' condition, i.e. the eggs and milt were exuding from the cloaca. Herring survived for only a short time out of water, and when dead they were treated in the following way: the scales and mucus were removed with a solution of 10% NaCl and 10% $\text{Na}_2\text{S}_2\text{O}_3$ and an antiseptic (tincture of iodine), was then applied. The body cavity was opened and the gonads drawn out. Part of the contents of the gonad was then cut out after removal of the surrounding epithelium and placed in a container, either dry or in the particular diluent. The testis tissue in the diluents was partly teased out. It was found best to spread the eggs on glass slides.

Immediately after each dissection, a control fertilisation was carried out with unstored spermatozoa and eggs. A test was also made to see how long the gametes remained fertile in the dead parents' body. Fertilisations of the stored gametes were carried out on successive days to a maximum of eight days, both eggs and spermatozoa being stored for the same length of time.

Counts of the percentage number of eggs fertilised were made under a binocular microscope at an hour later in most cases. Fertilised eggs were recognized by the development of a perivitelline space and a bipolar differentiation of the cytoplasm, as well as by their appearing more translucent than unfertilised eggs.

CONTROL FERTILISATIONS

Fertilisations immediately after death gave a percentage fertility of 85% to 100%.

The reduction in fertility when the gametes were left in the dead parents' body is shown in Figure 1. It shows that a 50% fertilisation may be obtained after about seven hours.

STORAGE OF EGGS

Herring eggs, which develop on the sea bottom, attach themselves to any suitable substratum by an adhesive substance once they have been wetted. This led at once to difficulties as the eggs stored in bulk in diluents stuck to each other, which made subsequent removal and fertility counts very difficult. The difficulty was overcome by spreading the eggs one layer thick on glass slides which were then placed in the diluent. It was obvious, however, that eggs stored in diluents became soft much sooner than they were stored dry.

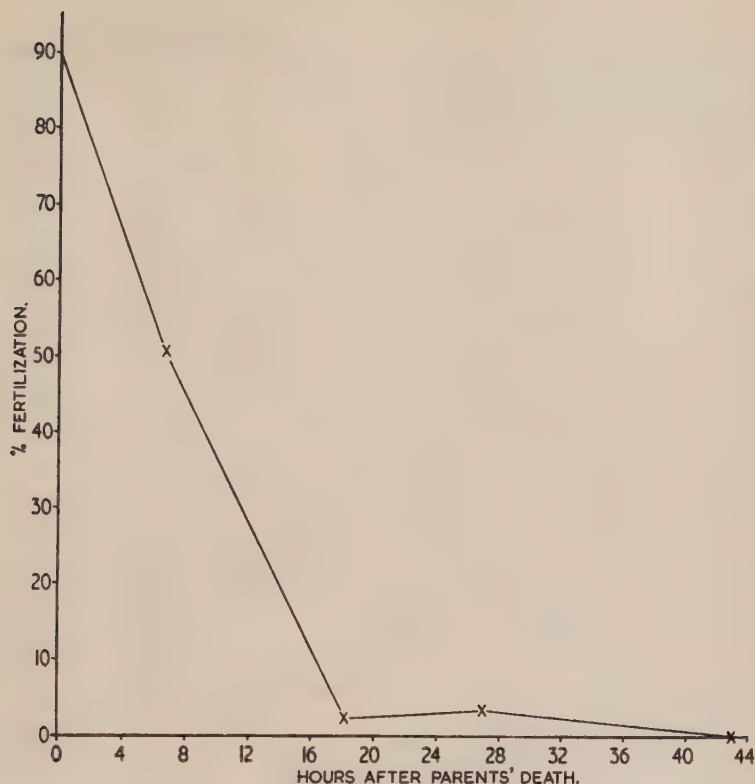


Fig. 1 The decline in fertility of gametes left within the dead parent's body.

A comparison of the fertility of eggs stored dry and in sea water every 24 hours is shown in Fig 2. The eggs were kept at a storage temperature of about 4° C and fertilised with spermatozoa which had been stored dry. Experiments, in which other diluents were used, are not reported here because the dry method of storage was obviously much more satisfactory.

The fertility after 24 hours is shown in Table I. The increased fertility, when dry storage is used, is statistically significant. In this and succeeding statistical tests fertilisations were compared after 24 hours as this was considered to be the maximum storage time required when the results were applied practically.

TABLE I

	Fertilised	Unfertilised	Total	% Fertilised
Eggs stored dry for 24 hours	191	32	223	85.7
Eggs stored in sea water for 24 hours . .	38	41	79	48.1
	229	73	302	

$$\chi^2=42.9 \text{ with d.f.}=1 \quad p < 0.01$$

STORAGE OF SPERMATOOA

The following fertility experiments on stored spermatozoa were carried out with the eggs stored dry.

(1) The use of dry compared with diluent methods. Fig. 3 shows the decline in fertility of spermatozoa stored under different conditions. The fact that dry storage is most effective after 24 hours is again shown by Table II,

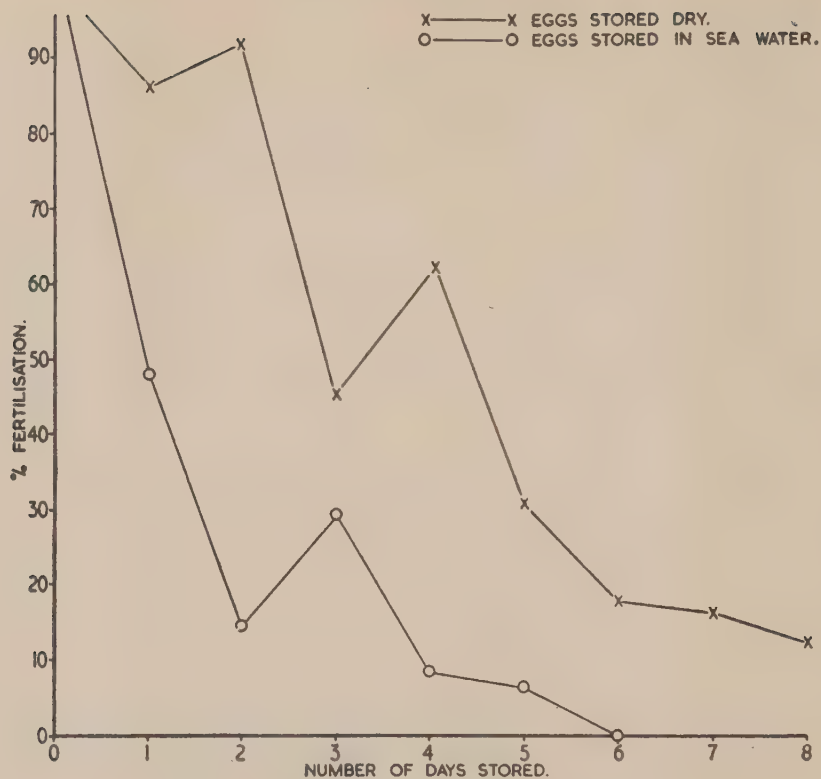


Fig. 2 The decline in fertility of eggs stored dry and in sea water.

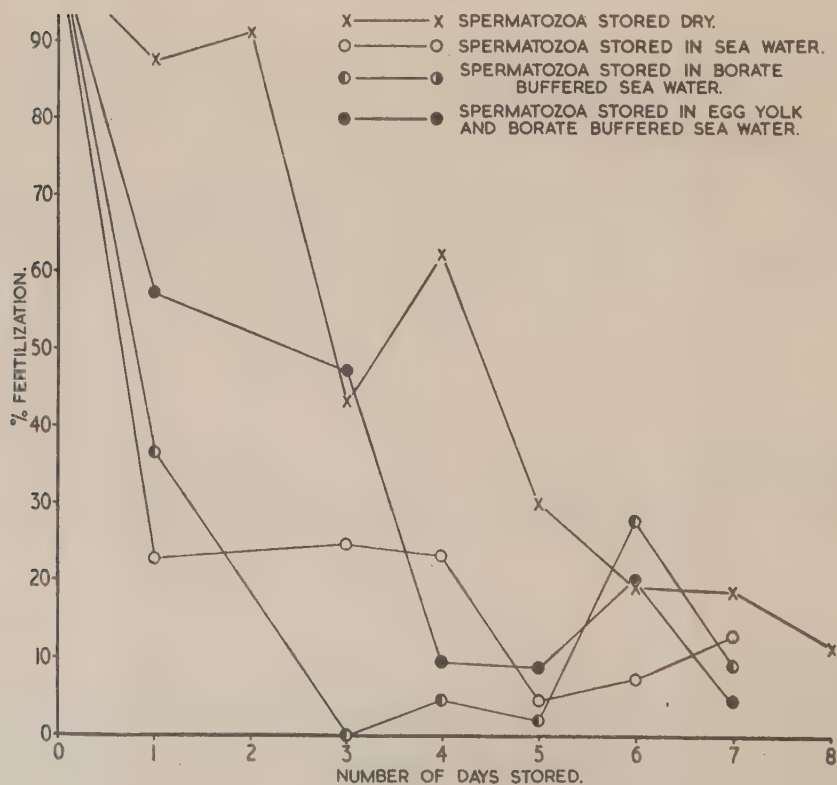


Fig. 3 The decline in fertility of spermatozoa stored under different conditions.

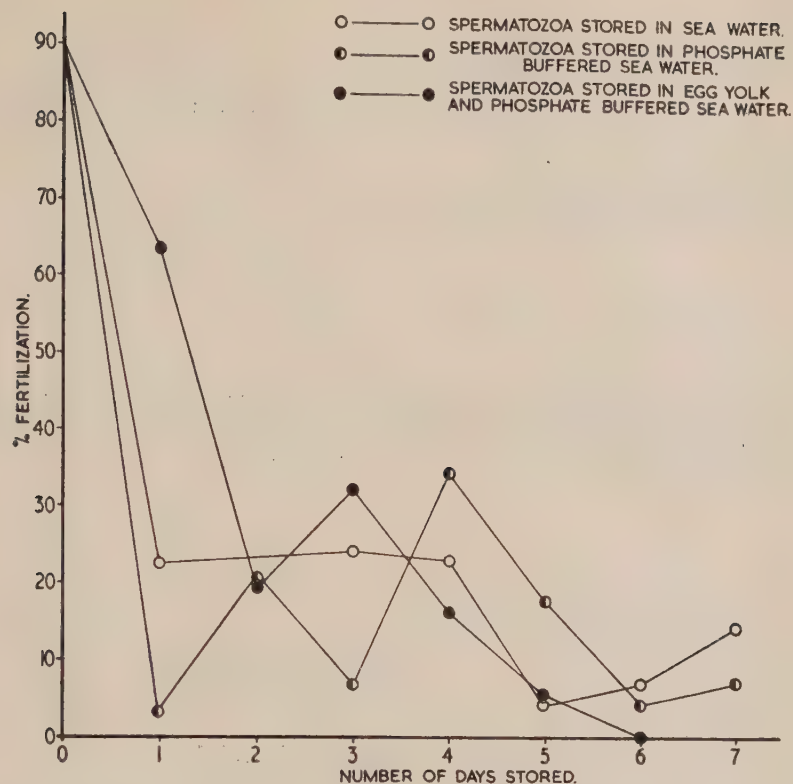


Fig. 4 The decline in fertility of spermatozoa stored using a borate buffer and egg yolk.

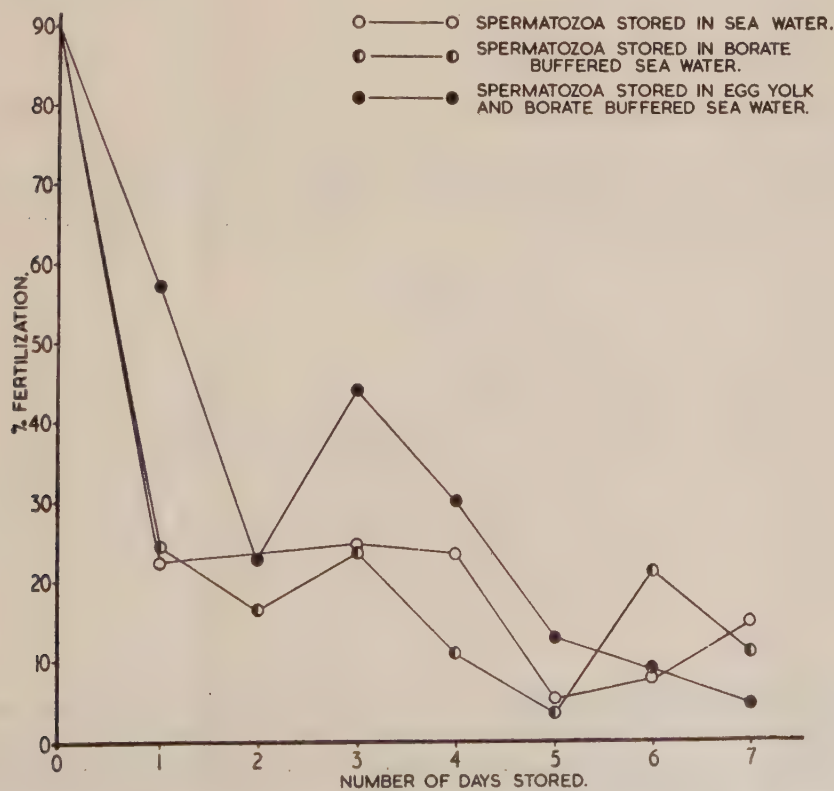


Fig. 5 The decline in fertility of spermatozoa stored using a phosphate buffer and egg yolk.

TABLE II

	Fertilised	Unfertilised	Total	% Fertilised
Spermatozoa stored dry for 24 hours ..	131	20	151	86.6
Spermatozoa stored in borate buffered sea water and egg yolk for 24 hours ..	32	24	56	57.1
	163	44	207	

$$\chi^2=19.5 \text{ d.f.}=1 \quad p < 0.01$$

(2) The use of egg yolk. The effectiveness of egg yolk as a diluent is shown in Table III and Figs. 4 and 5. It will be seen from Table III that after 24 hours the fertility in both buffered sea water plus egg yolk diluents is higher than in buffered sea water or sea water alone.

TABLE III

	Fert.	Unfert.	Total	% Fert.		Fert.	Unfert.	Total	% Fert.
Spermatozoa stored in egg yolk and borate buffer for 24 hours	32	24	56	57.1	Spermatozoa stored in egg yolk and phosphate buffer for 24 hours	40	24	64	62.5
Spermatozoa stored in borate buffer for 24 hours	44	139	183	24.0	Spermatozoa stored in phosphate buffer for 24 hours	3	143	146	2.1
	76	163	239			43	167	210	

$$\chi^2=20.3 \text{ d.f.}=1 \quad p < 0.01$$

$$\chi^2=96.28 \text{ d.f.}=1 \quad p < 0.01$$

From the graphs it can be seen that the advantage of using egg yolk is not so marked over long periods.

(3) The use of buffers. Figs. 4 and 5 also show the effect of using buffered sea water and sea water alone. The results of the fertilisation tests after 24 hours' storage in these media are shown in Table IV.

TABLE IV

	Fert.	Unfert.	Total	% Fert.		Fert.	Unfert.	Total	% Fert.
Spermatozoa stored in phosphate buffered sea water for 24 hours ..	3	143	146	2.1	Spermatozoa stored in borate buffered sea water for 24 hours	44	139	183	24.0
Spermatozoa stored in sea water for 24 hours ..	22	78	100	22.0	Spermatozoa stored in sea water for 24 hours ..	22	78	100	22.0
	25	221	246			66	217	283	

$$\chi^2=23.9 \text{ d.f.}=1 \quad p < 0.01$$

$$\chi^2=0.06 \text{ d.f.}=1 \quad p=0.86$$

It appears that after 24 hours sea water is as good a diluent as borate buffered sea water but that a phosphate buffer is less effective than sea water alone. This relationship is not maintained for long periods of storage, when it would seem that the use of either buffer gives a higher fertility than the use of sea water only.

(4) The effect of temperature. When it became obvious that dry storage gave the best results, an experiment was carried out to test the effect of a temperature nearer than of natural sea water. The usual storage temperature was 4° C; a temperature of 7° C was tried and was found to be less effective (see Fig. 6). This is confirmed by the data presented in Table V.

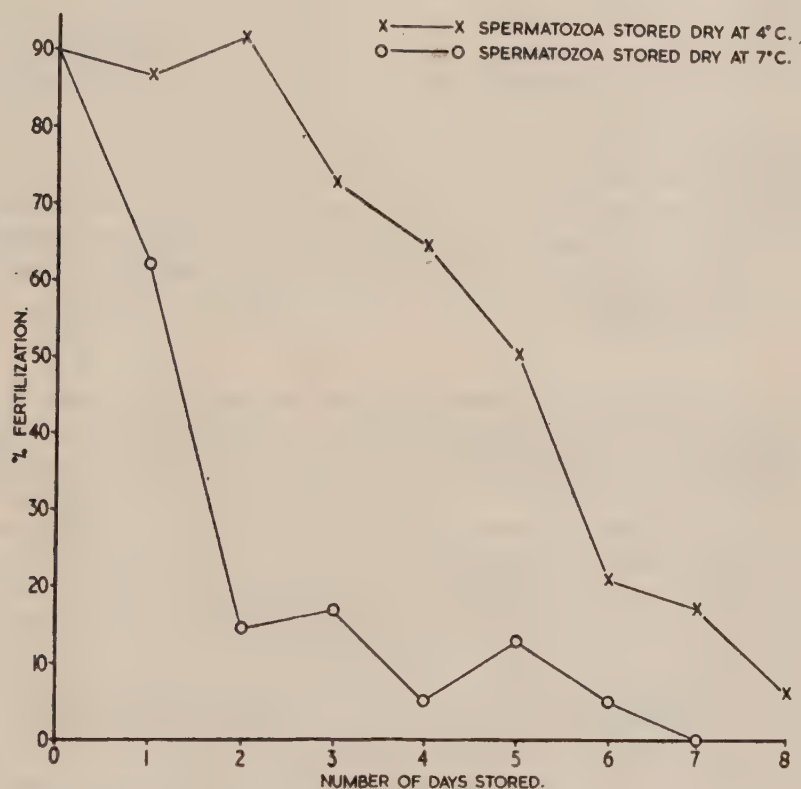


Fig. 6 The decline in fertility of spermatozoa stored dry at different temperatures.

TABLE V

	Fertilised	Unfertilised	Total	% Fertilised
Gametes stored dry at 4° C for 24 hours ..	131	20	151	86.6
Gametes stored dry at 7° C for 24 hours ..	37	23	60	61.7
	168	43	211	

$$\chi^2 = 15.2 \text{ d.f.} = 1 \quad p < 0.01$$

GENERAL

It has already been mentioned that both gametes were stored for the same length of time in these experiments, as ripe herring could not often be caught when required. On two occasions ripe females were caught after the beginning of an experiment so that it was possible to undertake fertility tests between stored and fresh gametes of both sexes. The results are shown in Table VI.

TABLE VI

		Days stored and % fertility							
		1	2	3	4	5	6	7	8
(1)	Both ♂ and ♀ stored dry	61.2	50.0	35.5	31.0	0	—	—	—
	♂ stored dry ♀ fresh	—	—	—	—	6.6	17.0	—	—
(2)	Both ♂ and ♀ stored dry	61.7	15.7	17.8	5.0	12.6	6.3	0	—
	♂ stored dry ♀ fresh	—	—	—	—	—	18.3	12.0	—

When a ripe male was caught seven days after the beginning of an experiment, no stored eggs were fertilised by the fresh spermatozoa.

No attempt was made in these experiments to hatch the fertilised eggs, but in subsequent experiments satisfactory hatching was obtained from eggs stored dry at 4° C. Herring gametes have been transported to Aberdeen from the Clyde, from Gairloch and Ullapool on the West Coast, from Shetland and from spawning grounds on the East Coast.

DISCUSSION

The carefully controlled conditions required in some fish rearing experiments should be applicable from the point of fertilisation. This work shows that there is no reason why ripe fish should not be caught at sea and their gametes stored until controlled conditions can be applied later in the laboratory.

The graphs do not show a steady decline in fertility with time and it is difficult to account for the sudden rise in fertility which occurs after some days' storage in many of the experiments. As the usual period of storage in practice did not exceed 24 hours the dry method is obviously the best, though high fertility was obtained in a few cases by other methods after longer periods up to seven days. Dry storage retains the spermatozoa in the undiluted state with a beneficial effect, but this may not be prolonged beyond a certain period of time.

The results of the egg yolk-buffer method are disappointing compared with the use of a buffer or oil. It is difficult, however, to compare the results with those obtained from artificial insemination for a number of following reasons. The requirements for storage of homoiotherm and poikilotherm spermatozoa are fundamentally different for only the former need protection against the 'cold shock' experienced when spermatozoa are transferred from high body temperature to low storage temperature. If the main property of egg yolk is to lessen the effect of 'cold shock,' it will be of little use where body and storage temperatures are similar. In the herring experiments both eggs and spermatozoa have been stored together whereas in the artificial insemination of mammals only the spermatozoa are stored. On the other hand the mammal has only one or a few eggs to be fertilised and these are relatively inaccessible, while in the herring the eggs are many and are easily accessible for fertilisation.

LONG TERM STORAGE

PREVIOUS WORK

As shown in the previous section, gametes will not live for much longer than a week at temperatures above freezing point. Gametes are usually killed by temperatures a few degrees below freezing point. This may be due to the formation of ice crystals causing mechanical injury or to the withdrawal of water as ice forms, upsetting the osmotic equilibrium of the cell.

Polge, Smith and Parkes (1949) first reported that fowl spermatozoa, stored in dilute glycerol at -79° C (in solid carbon dioxide), remained alive for as long as nine months. Polge and Rowson (1950) found that bull spermatozoa remained fertile after storage for nine months under these conditions.

This work suggested a means of storing herring gametes for long periods.

METHODS

Ripe herring were caught and treated in the same way as described previously. Various methods of storage and freezing in glycerol were tried.

(a) The testis tissue and eggs were placed in plastic-topped tubes which contained a solution of 12.5% glycerol w./w. in diluted sea water (one part distilled water to four parts sea water). After an interval of 30 minutes at a temperature of about 8° C the tubes were placed in solid carbon dioxide (-79° C) in vacuum flasks. (The flasks were kept in the cold store at Torry Research Station, Aberdeen. The author thanks the Superintendent for providing this facility). They were refilled with solid carbon dioxide once per week. Thawing after storage was carried out rapidly by immersing the tubes in water at about 40° C. The gametes were then transferred directly to sea water.

(b) Slow freezing was also tried. Instead of putting the tubes immediately into solid carbon dioxide they were put into 95% alcohol to which solid carbon dioxide was added piece by piece to give a cooling rate of 1 deg. C/min. Rapid cooling was introduced at about -30° C.

(c) An attempt was made to store the gametes by transferring them before freezing into gradually increasing concentrations of glycerol, i.e. in 5%, 10% and 25% glycerol in successive periods of 15 minutes. After freezing and thawing they were transferred to gradually decreasing quantities of glycerol before the final transfer to sea water.

(d) Storage of spermatozoa in 12.5% glycerol was tried at -28.9° C, the temperature of the cold store.

STORAGE OF EGGS

Eggs did not survive freezing for short or long periods and no method of storage could be found for them. No eggs could be fertilised after freezing and the yolk assumed a crystalline appearance.

STORAGE OF SPERMATOCYTES

Of the methods described above the first and most straightforward one gave the best results.

Spermatozoa stored in 12.5% glycerol and frozen rapidly to -79° C were found to be fertile on immediate thawing, after 14 days and after about six months. A comparison of slow and fast freezing showed that slowly frozen spermatozoa gave about 80% fertilisation and rapidly frozen sperm 85% fertilisation, when crossed with eggs from a different spawning type six months after freezing. The fertilisation rate in the controls, where both eggs and spermatozoa came from the same group, was not significantly different. It was not found necessary to transfer the spermatozoa into gradually increasing quantities of glycerol before freezing or into gradually decreasing quantities on thawing. Spermatozoa stored at -28.9° C were not fertile after six months.

GENERAL

This technique of storing spermatozoa has been used on three occasions for crossing spring and autumn spawning herring. The hatching rate has always been about 5% or less which was no lower than the controls where both parents were of the same spawning group.

DISCUSSION

A method of storing spermatozoa for long periods makes possible tests of interfertility which might be of value in a genetical study of related races and species of animals. It provides a means of breaking down "reproductive isolation" which is one of the chief criteria used in defining species (Mayr, 1942). If a positive infertility were found it would be a sign of genetical incompatibility. Complete fertility between the gametes would be a less valuable result as reproductive isolation might be occurring by other means. Thus while infertility between two animals is an indication of specific or greater difference, interfertility only enables one to say that reproductive isolation, if it exists, does not arise from failure of the spermatozoa to fertilise the egg.

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SUMMARY

- (1) Herring gametes have been stored dry and in various diluents for periods of up to eight days at a temperature of 4° C.
- (2) There was a general decline in fertility with time and it was found that the highest fertility was obtained when both eggs and spermatozoa were stored dry.
- (3) Herring spermatozoa have been stored in 12.5% glycerol at -79° C for periods of up to six months with a negligible loss of fertility. This has enabled crosses to be made between spring and autumn spawning herring. No satisfactory method of long term storage has been found for herring eggs.

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